SEPARATION AND DETERMINATION OF VITAMIN B_6 GROUP (PYRIDOXINE, PYRIDOXAL, PYRIDOXAMINE, PYRIDOXAL-PHOSPHATE AND PYRIDOXAMINE-PHOSPHATE)

SEPARATION BY PAPER ELECTROPHORESIS OF RIBOFLAVIN (RIBOFLAVIN, FMN* AND FAD) ANDOF NICOTINAMIDE (NICOTINAMIDE, DPN AND TPN) GROUPS

by

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The available methods for determining the vitamin B_6 forms, either individually, or together, are chemical, biological and microbiological[1].

The chemical methods are based on the formation of dyes, yielded by the condensation of the phenolic group which the B_6 compounds possess in the 3 position. However, these methods do not allow the determination of the individual components in a mixture[2].

This limitation is even more pronounced in biological assays, which involve the growth of rats maintained for a certain period with deficiency of the vitamin B_6 factors[3].

By the use of three microorganisms which respond differently to the three simple forms of the B_6 group (pyridoxine, pyridoxal and pyridoxamine), the individual determination of each of these compounds can be accomplished. By the application of such a procedure both before and after dilute acid hydrolysis (which cleaves the phosphate esters linkage) it is possible to get a reasonably accurate estimate of both pyridoxal-phosphate and pyridoxamine-phosphate[4].

WINSTEIN AND EIGEN[5] applied paper chromatography to the separation of the three unphosphorylated forms of vitamin B_6. This procedure, however, does not allow a completely satisfactory resolution between pyridoxine and pyridoxal; moreover it has not been applied to the phosphorylated forms.

Here we shall describe some electrophoretic procedures for separating and quantitatively determining all the forms of the vitamin B_6.

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* FMN = Flavin-mononucleotide (riboflavin-phosphate);
FAD = Flavin-adenine-dinucleotide;
DPN = Diphosphopyridine-nucleotide;
TPN = Triphosphopyridine-nucleotide.

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EXPERIMENTAL

Commercial samples (H. La Roche-Basel) of pyridoxine hydrochloride, pyridoxamine dihydrochloride and pyridoxal hydrochloride were used. Pyridoxamine-phosphate* was prepared according to PETERSON *et al.*[6] and pyridoxal-phosphate* according to VISCONTINI *et al.*[7].

Paper electrophoresis. A mixture containing about 10 μg of each of the compounds, pyridoxine, pyridoxal, pyridoxamine, pyridoxal-phosphate and pyridoxamine-phosphate, is applied in one spot on a paper (Munktell 20), halfway between the electrodes, the paper being first moistened in the buffer solution and pressed lightly between two filter papers to remove most of the liquid. The electrophoresis is run in acetate buffer pH 5.1, $\mu = 0.05$ and a current of 3.5 mA is applied over a 6–7 hours period.

For qualitative purposes the paper is placed between two glass plates which are firmly clamped together. On the completion of the run, the paper is dried in a fume chamber at room temperature and sprayed with the diazotized *p*-aminoacetophenone solution[8]. This solution is prepared as follows:

1. 3.18 g of *p*-aminoacetophenone dissolved in 45 ml of conc. HCl and diluted to 1 liter with distilled water.

2. Sodium nitrite 2.25 g/100 of distilled water.

3. Sodium acetate 25 g/100 ml of distilled water.

Two ml of 1, 10 ml of 2 and 10 ml of 3 are all mixed together immediately before use.

After spraying, the paper is dried for 10 minutes at 50–60°. The spots will appear differently coloured, with shades ranging from pink, through orange, to yellow. These colourations are stable. The separation of the compounds is illustrated in Fig. 1: Pyridoxal-phosphate and pyridoxamine-phosphate migrate towards the anode, the former compound having the greater mobility; pyridoxamine, pyridoxine and pyridoxal migrate, in decreasing order of mobility, towards the cathode.

There are some pyridoxal-phosphate preparations which, besides the main spot, recognizable as pure pyridoxal-phosphate, give another smaller spot in an intermediate position between pyridoxal-phosphate and pyridoxamine-phosphate. The colour of this spot, after spraying with the diazotized *p*-aminoacetophenone solution, appears to be the same as that given by the pyridoxine.

No breakdown of the phosphate esters occurs during the electrophoresis.

This electrophoresis separation can be usefully applied to the quantitative estimation of vitamin B_6 forms in a mixture. For this purpose the electrophoresis is run with the paper hanging in a closed chamber, without using the glass plates, at the same time maintaining all other preceding conditions. An accurately measured volume of the mixture to be analysed is applied on the paper from an Agla micrometer syringe (Burroughs Wellcome Ltd.). The spots are localized with a "Mineral-light" short-wave ultraviolet lamp and their identification is carried out from their position, by reference to the spots obtained in a parallel run of a known mixture of pure compounds. The elution with 0.05 *M* acetate buffer pH 5.1, is carried out, according to BRIMLEY AND BARRET[9], by collecting in graduated tubes 4 ml of the elution fluid, the extinction coefficients of which are determined in a Beckman spectrophotometer at 325 mμ[10]. Correction is made for the absorption due to the paper by using, as reference, the fluid collected by washing an identical strip of the same paper. Calculation may be based on the standard reference curves drawn from data obtained with solutions of pure compounds.

Starch column electrophoresis. A resolution of the vitamin B_6 compounds in larger amount was possible by use of the starch column electrophoresis.

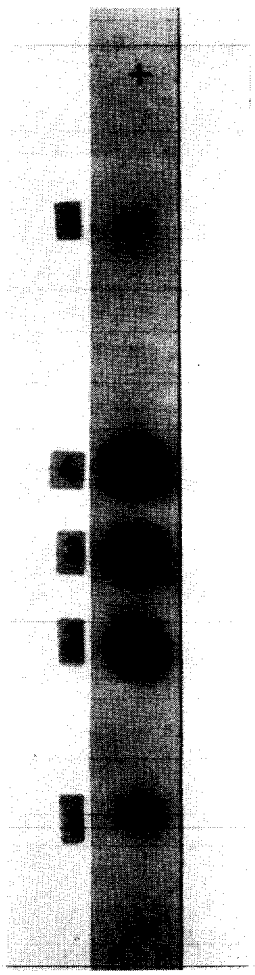

Fig. 1. Separation of a mixture of vitamin B_6 compounds by paper electrophoresis (acetate buffer pH 5.1, $\mu = 0.05$, 3.0 mA current during 5 hours). 1 = pyridoxamine; 2 = pyridoxine; 3 = pyridoxal; 4 = pyridoxamine-phosphate; 5 = pyridoxal-phosphate; 25 μg of each compound. The line indicates the origin of the initial spot.

* We are greatly indebted to Dr. H. A. SOBER for a sample of crystalline pyridoxamine-phosphate and to Prof. M. VISCONTINI for a sample of pyridoxal-phosphate.

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TABLE I

RECOVERY OF PYRIDOXINE, PYRIDOXAL, PYRIDOXAMINE, PYRIDOXAL-PHOSPHATE AND PYRIDOXAMINE-PHOSPHATE FROM A MIXTURE SEPARATED BY ELECTROPHORESIS ON PAPER

	Applied (μg)	*Recovered (μg)*	*Recovery (%)*
Pyridoxine	74.8	76.2	101.9
	74.8	74.7	100.0
	74.8	75.0	100.3
Pyridoxal	64.2	62.5	97.3
	64.2	67.0	104.5
	64.2	63.0	98.1
Pyridoxamine	53.6	52.0	97.0
	53.6	51.8	96.6
	53.6	52.2	97.3
Pyridoxal-phosphate	48.0	44.1	91.9
	48.0	44.8	93.3
	48.0	45.5	94.8
Pyridoxamine-phosphate	52.0	48.3	92.9
	52.0	47.1	90.6
	52.0	49.0	94.2

The apparatus design and the technical details were according to FLODIN AND PORATH[11]. As in the paper electrophoresis procedure acetate buffer pH 5.1, μ = 0.05 was used. A mixture of pyridoxine, pyridoxal, pyridoxamine, pyridoxal-phosphate and pyridoxamine-phosphate (2 mg of each compound) in 2 ml of the acetate buffer is applied at the top of the column (50 × 3cm). Fifty-five ml of the buffer are allowed to flow down before applying the current. The top of the column is connected to the cathode, the bottom to the anode. A current of 18 mA is applied for 14 hours. On completion of the run, the column is disconnected from the electrophoresis apparatus and adapted to the fractions collector. The elution with the acetate buffer is adjusted to give a flow rate of about 20 ml/hour and the effluent is collected in a series of 3 ml fractions, the extinction coefficients of which are measured in the Beckman spectrophotometer at 325 mμ. By means of standard reference curves, drawn from data obtained with solutions of the pure compounds, the concentration of the compounds, expressed in millimoles (mM), is then calculated.

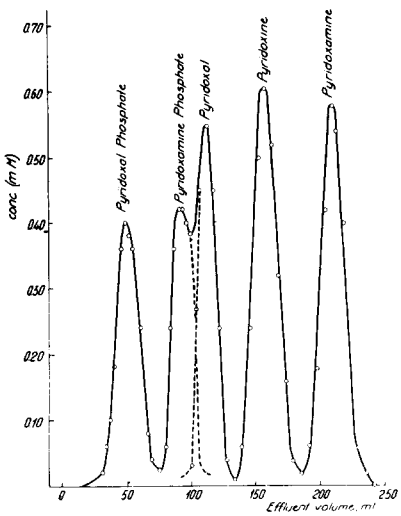

Fig. 2. Separation of vitamin B_6 forms from a mixture, containing 2.6 mg of each compound, *by starch column electrophoresis* (acetate buffer pH 5.1, μ = 0.05, 18 mA during 14 hours). The dotted line indicates the separation when pyridoxamine-phosphate or pyridoxal is omitted from the mixture.

The identification of the compounds is carried out with the help of the paper electrophoresis as previously described. Since the presence of the acetate buffer markedly alters the position of the spots on the paper electrophoresis, it is necessary to desalt the solution beforehand. This is achieved by the adsorption from the buffered solution of the B_6 compounds on partially deactivated carbon and eluting them with a mixture of ethanol, acetone and water (1 : 1 : 1). The partial deactivation of the carbon is required because the adsorption of the B_6 compounds on pure carbon is not completely reversible. Pretreatment of the carbon (Carbo Activ) with a saturated solution of hexanol in water (0.5 %) is suitable for this purpose: hexanol solution is percolated through the carbon in the proportion of 50 ml for each gram of carbon. The adsorption of the B_6 compounds is seriously impaired by a stronger deactivation if the carbon is treated with a larger amount of hexanol.

When desalted the fractions can be conveniently evaporated under reduced pressure at about 40° until the favourable concentration for paper electrophoresis is attained.

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Differential adsorption of pyridoxine, pyridoxal and pyridoxamine on the starch. When determining the dead volume for the B_6 compounds on the starch column, in connexion with the electrophoresis experiments described above, a differential adsorption of the free forms (pyridoxine, pyridoxal and pyridoxamine) on the starch was noticed. Thus it was possible to achieve a fairly good separation of these three compounds by simple chromatography on a starch column (50 × 3 cm), using as solvent the same acetate buffer (0.05 *M*, pH 5.1) as in the electrophoresis experiments. A resolution of all the five forms of vitamin B_6 was not possible by this procedure.

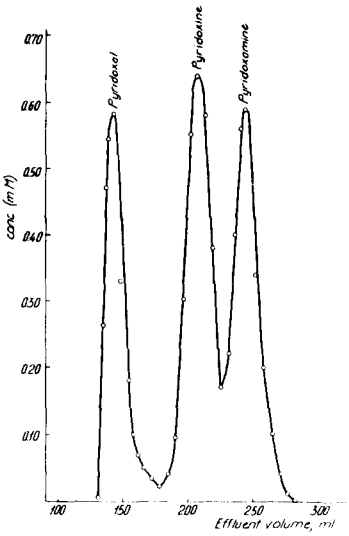

Fig. 3. Chromatographic separation on starch column (50 × 3 cm) of a mixture containing 2.6 mg of each compound. Solvent = 0.05 *M* acetate buffer at pH 5.1.

SEPARATION BY PAPER ELECTROPHORESIS OF RIBOFLAVIN AND ITS COENZYMES (FMN AND FAD)

Under the same experimental conditions previously described for the paper electrophoresis of the Vitamin B_6 group (acetate buffer pH 5.1, $\mu = 0.05$, 3 mA during 7 hours), it was possible to achieve a good separation of riboflavin and its coenzymes (FMN and FAD). Riboflavin was a commercial sample, FMN was prepared according to VISCONTINI *et al.*[12] and 60% pure FAD was kindly supplied by WHITBY[13]. 0.6 μg of riboflavin, 1 μg of FMN and 5 μg of FAD have been used to obtain the separation represented in Fig. 4; however smaller amounts can be detected very easily.

The spots are localized in U.V. light by their bright yellow fluorescence and their identification is effected by reference to the spots obtained in a parallel run of the single compounds.

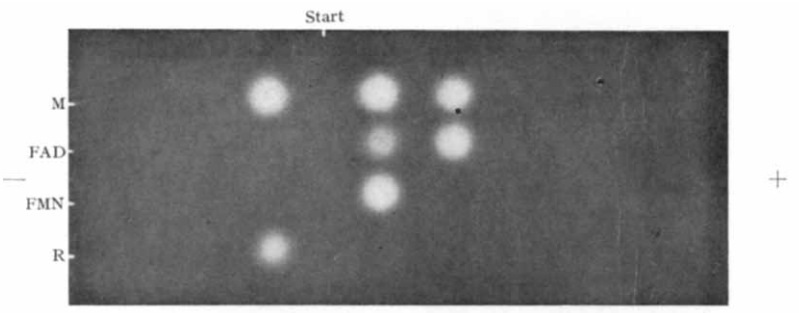

Fig. 4. Separation of vitamin B_2 compounds from a mixture containing 0.6 μg of riboflavin (R), 1 μg of FMN and 5 μg of FAD, by *paper electrophoresis* (acetate buffer pH 5.1, $\mu = 0.05$, 3.5 mA during 7 hours). The photograph was taken in U.V. light using panchromatic plates and a yellow filter on the objective; the exposure time was 30 seconds.

SEPARATION BY PAPER ELECTROPHORESIS OF NICOTINAMIDE AND ITS COENZYMES (DPN AND TPN)

Under the above experimental conditions (acetate buffer pH 5.1, $\mu = 0.05$, 3.5 mA current for 7 hours) it became feasible to separate nicotinamide and its coenzymes

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(DPN and TPN). Nicotinamide, DPN and TPN were commercial samples (Sigma Co.). 15 μg of each compound have been used in the experiment represented by Fig. 5. On the completion of the run, the paper is sprayed with a mixture of 2% $Na_2S_2O_3$ and 4% $NaHCO_3$ (1:1) in order to transform DPN and TPN to their dihydro-forms[14]. On examination of the paper by short-wave U.V. light, DPN and TPN, in the dihydro-forms, appear as spots with feeble fluorescence, while nicotinamide and the compounds, accompanying TPN as impurities, will appear as dark spots.

One of the spots assumes the same position as ADP, run in parallel, and, therefore, may be identified, presumably, with this compound. The identification of the other impurity accompanying TPN was not investigated.

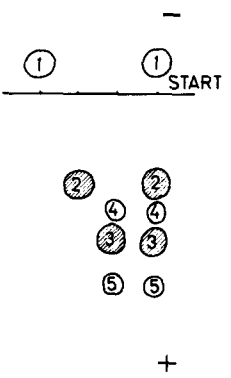

Fig. 5. Separation of a mixture containing 15 μg of each compound by paper electrophoresis (acetate buffer pH 5.1, $\mu = 0.05$, 3.5 mA during 7 hours). 1 = nicotinamide; 2 = DPN; 3 = TPN; 4 = ADP(?); 5 = unknown substance.

SEPARATION BY PAPER ELECTROPHORESIS OF THE B_2, B_6 AND NICOTINAMIDE GROUPS COMBINED IN ONE MIXTURE

Since the described separation of each B_2, B_6 and nicotinamide group is realized under the same experimental conditions, an attempt was made to separate all these factors from one mixture. The mixture used consisted of 15 μg of each of the compounds, pyridoxine, pyridoxal, pyridoxamine, pyridoxal-phosphate, pyridoxamine-phosphate, nicotinamide, DPN and TPN, together with 3 μg of each of the compounds, riboflavin, FMN and FAD. With the exception of the duration of the current, the same previous conditions were maintained (acetate buffer pH 5.1, $\mu = 0.05$, current of 3.5 mA *for 10 hours*). The localization and the identification of the spots have been effected as follows:

The paper is first examined by means of a "Mineral-light" lamp, which reveals individually all the spots and permits the identification of the spots corresponding to riboflavin, FMN and FAD by their yellow fluorescence. After spraying with the dithionite-bicarbonate mixture, the examination with the "Mineral-light" lamp, fitted with a double blue filter, reveals the spots of DPN and TPN, which are now fairly fluorescent. Finally by spraying with the diazotized *p*-aminoacetophenone, the spots of the vitamin B_6 group are readily localized and identified.

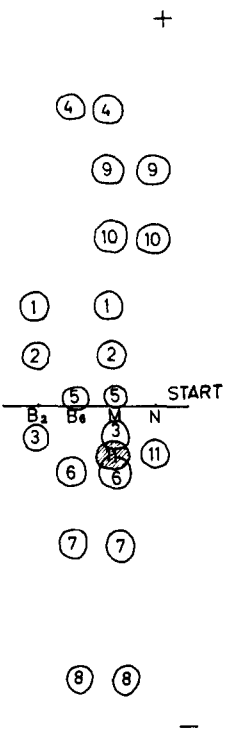

Fig. 6. Separation of B_2, B_6 and nicotinamide groups, combined in one mixture, by paper electrophoresis (acetate buffer pH 5.1, $\mu = 0.05$, 3.5 mA during 12 hours). 1 = FAD; 2 = FMN; 3 = riboflavin; 4 = pyridoxal-phosphate; 5 = pyridoxamine-phosphate; 6 = pyridoxal; 7 = pyridoxine; 8 = pyridoxamine; 9 = TPN; 10 = DPN; 11 = nicotinamide.

Ten of the 11 examined compounds are very easily identified; because it is partially obscured by the riboflavin and pyridoxal spots, nicotinamide can escape the identification.

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DISCUSSION

The procedures described here are much simpler than previous methods and can be applied successfully in the separation and determination of the B factors considered in this report. These procedures also have the advantage of removing the impurities accompanying some factors. In these experiments pyridoxal-phosphate has been separated from a contaminating substance, which appears to be pyridoxine-phosphate. FAD has been separated from the accompanying FMN, which seems to be the only contaminating substance in the sample received from Dr. WHITBY. From a commercial sample of TPN, DPN, as well as two other substances, has been separated, one of the latter may be ADP. Through the above procedures these factors, previously unobtainable in pure form, may be now readily available, although in fairly small quantity. In a mixture containing all the 11 compounds considered in this paper, at least 10 can be distinguished by paper electrophoresis; the nicotinamide spot is partially obscured by the presence of the pyridoxal and riboflavin spots.

By means of the starch electrophoresis, which has been applied only to the B_6 group, it is possible to separate larger amounts of each product and the technique can be used also for preparative purposes. With this procedure, however, a complete quantitative resolution between pyridoxamine-phosphate and pyridoxal was not achieved satisfactorily. Nevertheless a complete quantitative separation of the remaining 4 compounds can be obtained if the fifth, pyridoxamine-phosphate or pyridoxal, is omitted from the mixture. It has been observed that the unphosphorylated forms of the B_6 group are differently adsorbed on the starch, so that a partial separation of them is possible by simple chromatography on the starch column.

Since these compounds are adsorbed on the starch with increasing power, according to their positive charge strength (pyridoxamine > pyridoxine > pyridoxal), the application of electrophoresis further separates these substances to give a complete resolution. The column electrophoresis, with a cellulose powder as support, did not give a satisfactory resolution of the B_6 compounds; similarly the same cellulose powder does not show any differential adsorption for the compounds of this group.

ACKNOWLEDGEMENT

The authors wish to express sincere thanks to Professor ARNE TISELIUS for the privilege of working at his Institute and for all his help and advice. The expenses in connection with this investigation have been defrayed by grants to the Institute of Biochemistry, University of Upsala, from the Rockfeller and Wallenberg Foundations.

SUMMARY

The separation and determination of vitamin B_6 forms (pyridoxine, pyridoxal, pyridoxamine, pyridoxal-phosphate and pyridoxamine-phosphate) with paper electrophoresis and starch column electrophoresis are described.

Procedures for separating riboflavin derivatives (riboflavin, FMN and FAD) and nicotinamide derivatives (nicotinamide, DPN and TPN) by paper electrophoresis are reported.

A separation of all the above compounds from one mixture has also been obtained.

RÉSUMÉ

Description de méthodes permettant la séparation et la détermination de diverses formes de vitamine B_6 (pyridoxine, pyridoxal, pyridoxamine, pyridoxal-phosphate et pyridoxamine-phosphate) à l'aide d'électrophorèse sur papier et d'électrophorèse sur colonne d'amidon.

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Des procédés pour séparer les dérivés de la riboflavine (riboflavine, FMN et FAD) et les dérivés de la nicotinamide (nicotinamide, DNP et TPN) par électrophorèse sur papier, sont également décrits.

Les auteurs ont également réussi la séparation de toutes les substances décrites ci-dessus à partir d'un mélange.

ZUSAMMENFASSUNG

Es wird die Abtrennung und Bestimmung von Vitamin B_6-Formen (Pyridoxin, Pyridoxal, Pyridoxamin, Pyridoxalphosphat und Pyridoxaminphosphat) durch Elektrophorese mit Papier und Stärke-säulen beschrieben.

Es werden Verfahren zur Abtrennung von Riboflavinderivaten (Riboflavin, FMN und FAD) und Nicotinamidderivaten (Nicotinamid, DPN und TPN) durch Papierelektrophorese berichtet.

Ebenso konnte eine Trennung aller obengenannten Verbindungen aus einer Mischung erreicht werden.

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Received January 13th, 1954